{ WHITE PAPER }

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Minimising Assay Bias Using Standards, Controls and Automated Liquid Handling

Tim Stroud & Chloe Carter
Titian Software

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ABOUT THIS WHITE PAPER

This white paper draws on Titian Software's long running partnerships with multiple vendors of laboratory automation and its extensive experience of implementing sample management systems for organisations of all sizes, plus the author's 20+ years experience in the pharmaceutical and software industries.

INTRODUCTION

When constructing and running assays, a lot of effort goes into removing bias from assay data. Wherever possible, the aim is to treat all test samples in the same manner, at the same time, using the same equipment, to reduce variation and sources of error in the data gained from the assay. In an ideal assay, the only variation would be caused by the samples in the wells.

Removing bias in assays is essential because "that results in a biological method that can be applied to test compounds over a period of weeks, months, or years and for which the results generated at the outset will be comparable to every test along the way." [1]
If your assay method is biased, then all the data generated from these assays will also be compromised.

In this white paper, we look at the reasons why assay plate Controls and Standards are used and how these need to be managed to ensure they provide a reliable measure of data quality.

MINIMISING BIAS IN ASSAYS

Because the assay ideal is hard to achieve, scientists use Controls and Standards to measure an assay's consistency and check that its data may be relied on. The criteria for standards and controls are:

- A control compound (or a control vaccine, antibody or other therapeutic) is a single concentration. Typically, both 100% effect (e.g. top dose of a compound) and 0% effect (e.g. diluent only) are used to determine the assay window. The response of your dilution series test compound should lie between these two points.
- A standard or reference is a well-characterised substance that responds the same way every time or within a certain range. It should run from your 0% effect to your 100% effect dose throughout your plate, allowing you see if your assay is working consistently.

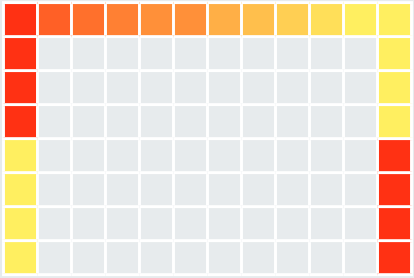
Ideally, your test samples, controls and standards all go through the same process at at the same time, using the same instrumentation. This is not always possible, so adding standards and controls must be carefully planned, tracked, managed – and auditable. It is a case of 'Who watches the watchman?' Standards and controls are there as a quality control (QC) check of assay data, but you need to ensure that they too are added consistently and in a way which will provide a reliable measure for assay data.

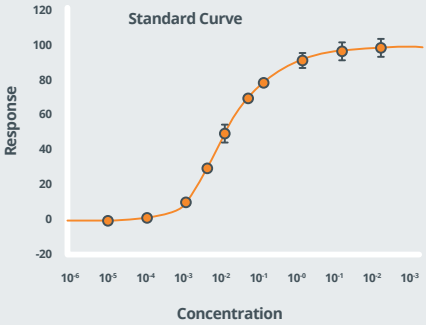
While your Laboratory Information Management System (LIMS) or sample management software will be able to reliably track sample additions and provide an audit trail for these, surprisingly, the handling of controls and standards is often overlooked. Most commonly, controls and standards are not tracked by the LIMS, but instead defined at the analysis stage and thus have no audit trail. Fortunately, some LIMS do have the capability to track the addition of controls and standards. Titian's Mosaic software is one.

HOW CONTROLS VALIDATE AN ASSAY: STANDARD CURVE

The difference in signal (e.g. fluorescence, luminescence, colourimetric, radioactive decay) between the Max and Min controls is your assay window. Generally, the larger the assay window the better. A small assay window will require a small standard deviation whereas with a large assay window you can accept a larger degree of variation.

The responses to the standard curve data points are expressed as percentage based on the Max and Min control responses. Standard curves are typically plotted with concentration on the logarithmic X-axis and the % responses on the Y-axis. The slope of the curve should be between 0.8-1.2.





HPE	100% = Average Hundred Percent Effect
ZPE	0% = Average Zero Percent Effect

The Z-prime (Z') value, based on the mean and standard deviations of the Max and Min control wells, takes the assay window AND variation into account.

Z-prime can never go above 1:

- 1.0 the best it can be
- 0.6-1.0 an excellent assay
- 0.0-0.5 a marginal assay
- Less than 0 means there is too much variation and overlap between the Max and Min controls

From the standard curve you can extrapolate an IC/EC_{50}, that should be within the expected range.

The standard curve must pass validation to give confidence in the results gained for the test substances.

POSITIONING CONTROLS AND STANDARDS ON ASSAY PLATES

Controls and standards should be present on every plate to check for plate-to-plate variations. If not, you may be faced with the following compromises:

- Plates with fewer wells (96) mean you either use fewer standards and controls on the plate to provide your quality check or you limit the number of test samples you can run per plate.
- If your controls and standards are spread thinly across multiple plates, or their positions randomised, you may not have sufficient quality control data to reliably assess each plate.
- If there are plates that don't have controls and standards, how will you confirm that the assay data from these plates is acceptable?

Careful placement of your controls and standards can also be critical to avoiding:

- Interactions between your 100% effect controls and reads for experiment sample measurements in adjacent wells.
- Edge effects caused by evaporation or CO_2 concentration variations across the plate.

Conventional liquid handling means controls and standards are most easily added in columns, commonly 1 and 24, making them subject to the effects described above. Some companies use the flexibility of acoustic dispensers to position controls and standards throughout the plate in a serpentine plate pattern to avoid these problems. However, it is important to review the workflows for your preferred approach. Sometimes the limitation may be whether your automation is physically capable of carrying it out in the best way.

HOW LIQUID HANDLING AFFECTS CONTROLS AND STANDARDS

Because controls are a single concentration, they are easier to process. The operator can transfer them to an assay plate as they are. However, standards are usually supplied at a single top concentration which needs to be serially diluted across the plate to generate a dose response curve, preferably at the same time as the test samples.

Different liquid handler types require different approaches to preparing assay plates. They may also introduce bias in different ways. This creates processing challenges for the sample management team who must weigh up pros and cons of each approach and track which was used for each assay addition. For example:

- **The closest to ideal process** is to cherry pick the test samples and standard in the top doses, then serialise both together at the same time across the plate. However, liquid handlers with this flexibility are not as fast at processing bulk requests.
- **Some liquid handlers do not support serialisation** or the separate placing of standards. These require a pre-prepared intermediate assay plate which can be stamped out. This process is often used for HTS runs as it is very fast, although it means standards and controls are added at a different time to your test samples.
- **Acoustic dispensers** are very flexible for positioning standards and controls and creating dose response plates. However, they have only one liquid dispense transducer, so they are not as fast as some systems. Acoustic source plates have limited volumes which may restrict the number of plates that can be prepared in one run.
- **Pre-made controls and standards plates.** Due to different liquid handler abilities, it is common for a compound management team to stamp out a stock of control and standard plates, which they use for a few weeks. However, this means that while your test compounds are freshly serialised, your standards may be two weeks old and the plate unpeeled several times, which will introduce bias.
- **Adding controls and standards just in time for the assay.** This might be due to an unstable substance; or because multiple copies of a plate are made to optimise sample processing but will be used in different assays and so require different standards or controls. The compromise here is that the standards are added at a different time to your test samples, using different pipettes and probably a different liquid handler.

MANAGING CONTROLS AND STANDARDS

Apart from different liquid handler processes and the timing of additions, some of the challenges in managing controls and standards include:

- Calculating the amounts of each standard and control required and comparing this to source volumes, so you know if you can process all the plates needed from one source.
- If multiple sources are needed, managing the scheduling so that you know how many plates can be processed from the first tube, from the second, and so on.
- Pre-preparing controls and standards in different plate layouts for multiple assays and then tracking these to ensure each layout is used for the right assay.
- Ensuring liquid handler tips are set up correctly for the pre-prepared plate layout you are using.
- Tracking the different dead volumes of each pipette tip type and labware type used so you can factor in these losses when calculating the amounts of standards and controls required.
- Creating an efficient workflow when adding your controls and standards. This includes avoiding repeating automation steps if they can be done in a single run at the same time; and maintaining sample integrity by avoiding exposing a plate for longer than necessary or reopening sealed plates.
- Maintaining an audit trail that includes the addition of controls and standards, which may be essential to refer to when assessing whether, for instance, variable data from a plate was caused by a liquid handling error

Managing these different approaches, equipment and variables is made easier if they can be automatically tracked by sample management software or LIMS.

CONTROLS AND STANDARDS IN COMPLEX ASSAYS

So far, we have discussed simpler examples of managing controls and standards in single sample wells. More complex assays, such as those using combination screening or pooling, lead to much greater challenges.

For instance, in a pooled substance assay, one or more additives may be dispensed into each mixture well to validate each well measurement with a pool QC sample. This means a much greater number of substances needs to be tracked for every individual well used, as well as assessing total volumes of each substance needed, recording how each one is added and from where.

Again, the challenge is to schedule and track all the sources, volumes and transfers required to create such plates and also record the complex interaction of potentially different numbers of samples in each pool plus the subsequent sample and solvent concentrations.

CONCLUSION

Managing controls and standards to provide a rigorous quality check of assay data is central to ensuring your assay results are reliable and can be compared meaningfully with similar screens.

It requires you to manage lab automation and processes with as much care and attention as is given to choosing chemically suitable substances as standards and controls and how these are placed. It is a significant undertaking and one that is often undervalued. It is made significantly easier with a good sample management LIMS, such as Titian's Mosaic software, that will manage workflows, tracking and provide an audit trail for your controls and standards as well as your samples.

Effect on assay bias	Pros	Cons
Cherry picking liquid handlers	Close to ideal: standard and sample serialised together during the same run as the test samples are being processed	Can be slower
Fixed head liquid handlers	Fast stamping out of large numbers of plates and very good for panel screening due to lack of variation between plates	Pre-laid out control/ standards plates are often used which may introduce bias Complexity for setting up tips for dispensing controls and standards at separate times
Acoustic dispensers	Ultimate flexibility for positioning controls and standards Increased accuracy provides good data quality	Acoustic Dose Response (e.g. EDR) workflows need to create intermediate dilution plates to produce a serial curve Standards should be fulfilled via the same intermediates for consistency and avoid bias Can be slower
Pre-made plates containing standards and controls	Fast and efficient processing for bulk creation of plates with the same layout	Subject to bias as may be made well ahead of when test samples are added Different equipment may be used for these two processes
Just in time addition of standards	Essential for unstable reference substances Efficient when supplying similar plate copies to panels of screens	Time delay between processing samples and standards may introduce bias Different equipment may be used for these two processes bringing in further variation

Reference

1. Janzen, B ; Screening Technologies for Small Molecule Discovery: The State of the Art ; Chemistry & Biology, Vol 21 Issue 9, 2014; https://www.sciencedirect.com/science/article/pii/S1074552114002440

AUTHORS:

TIM STROUD

After 15 years as a scientist at Pfizer working across many different groups, Tim Stroud moved to MedImmune for 3 years to specialise in managing laboratory automation. He joined Titian Software in 2014 and is now a Sales and Application Consultant.

CHLOE CARTER

Chloe has been writing for technology and life science companies for 25 years

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info@titian.co.uk

www.titian.co.uk

UK Tel:
+44 20 7367 6869

USA Tel:
+1 508 366 2234